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Zinc reverses glycine-dependent inactivation of NMDARs in cultured rat hippocampal neurons

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In the presence of glutamate and co-agonists, e.g., glycine, the N-methyl-D-aspartate receptor (NMDAR) plays an important role in physiological and pathophysiological brain processes. Previous studies indicate glycine could inhibit NMDAR responses induced by high concentration of NMDA in hippocampal neurons. The mechanism underlying this inhibitory impact, however, has been unclear. In this study, the whole-cell patch-clamp recording and Ca^{2+} imaging with Fluo-3/AM under laser scanning confocal microscope were used to analyze the possible involvement of NMDAR subunits in this effect. We found that the peak current of NMDARs and Ca^{2+} influx induced by high concentration of NMDA were reduced by treatment of glycine (0.03–10 μmol L^{-1}) in a dose-dependent manner, and that the glycine-dependent inhibition of NMDAR responses, which were induced at 300 μmol L^{-1} NMDA, was reversed by $ZnCl_2$ through the blocking of the NR2A subunit of NMDARs, but was less influenced by ifenprodil, a NR2B inhibitor. Our results suggest that the glycine-dependent inactivation of NMDARs is potentially modulated by the regulatory subunit NR2A.

N-methyl-D-aspartate (NMDA), NMDARs (NMDARs), glycine, zinc, inactivation, hippocampal neurons

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N-methyl-D-aspartate receptor (NMDAR) ion channels are generally considered as postsynaptic targets of glutamate-mediated synaptic transmission in the central nervous system. NMDARs play critical roles in physiological processes such as synaptic plasticity and memory by allowing glutamate-gated calcium influx into neuronal cells [1]. A property of the NMDARs is its voltage-dependent activation, a result of ion channel block by extracellular Mg^{2+} ions, which allows the flow of Na^{+} and small amounts of Ca^{2+} ions into the cell and K^{+} out of the cell [2]. Besides, the NMDAR requires co-activation of the glycine site located on its NR1 subunit by D-serine or glycine in addition to the synaptically released neurotransmitter glutamate [3,4]. However, the mechanistic connection between glutamate and glycine binding in the modulation of NMDAR activation and inactivation is unclear.

Many mechanisms have been proposed to account for the mediation of the inactivation of NMDARs, including glycine-dependent desensitization, glycine-independent desensitization [5,6], Ca^{2+}-dependent inactivation [7–9] and surface membrane receptors internalization [10]. Activation of NMDARs that have been pre-equilibrated with glycine produces a peak current, which decays or desensitizes to a steady-state level at high concentrations of NMDA. According to the desensitization of currents in the presence of saturation concentrations of glutamate and glycine agonists, two types of desensitization, glycine-independent or glycine-dependent, have been characterized for NMDARs.

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Magnitude of glycine-independent desensitization and calcium dependent inactivation are controlled by the key residues in the NR2 subunit [11]. The degree of desensitization is inversely dependent on the saturation degree of the glycine binding site and results from a weakening of glycine affinity upon channel activation [6,12]. The mechanisms underlying desensitization or inactivation of NMDAR are complicated. Nong *et al.* [10] raise the possibility that NMDARs may undergo regulated transport to and from the cell surface. We previously proposed a new mechanism of glycine-dependent inactivation, which occurred when NMDARs were activated by high concentrations of NMDA (optimal 300 μmol L^{-1}) via activation of the glycine binding site of NMDARs but not activation of the strychnine-sensitive glycine-receptor-gated chloride channel [13]. The inhibitory influence of glycine on NMDARs provides a new insight into the complexity of neuronal feedback loops and synaptic transmission.

However, the NMDAR forms a heterotetramer between two NR1 and two NR2 subunits [14,15], whether NR2 subunits of NMDARs are associated with the glycine-dependent inactivation and contribute to the inhibition of glycine affinity or not is to be answered. The purpose of this study was to investigate whether and how the inhibitors of NR2A or NR2B, i.e., zinc or ifenprodil, influenced glycine-dependent inactivation in cultured hippocampal neurons.

1 Materials and methods

1.1 Animals and chemicals

Sprague-Dawley rats were obtained from the Experimental Animal Center of Nantong University, Jiangsu, China. All procedures used in this study were in accordance with our institutional guidelines, which comply with international rules and policies. Common inorganic salts were purchased in China, chemicals for neuronal culture were products of GIBCO (Invitrogen Corporation, Carlsbad, USA), Fluo3-AM from Dojindo Laboratories (Kumamoto, Japan), other chemicals except those indicated elsewhere were purchased from Sigma-Aldrich Corporation (Saint Louis, USA).

1.2 Cell culture

The procedure for producing low-density rat hippocampal neuronal cultures was according to Stefanie detailed previously [16]. Briefly, the hippocampi were isolated from the embryonic (E17–18) rats, cut into pieces, and digested with 0.125% trypsin at 37°C for 12 min. The digested brain tissue pieces were mildly triturated to a single-cell suspension, and plated at a density of 75000 cells mL^{-1} on to coverslips (1 cm×1 cm) coated with poly-L-lysine overnight at 4°C prior to the experiment. The day of plating was counted as day-in-vitro (DIV) 0. The feeding medium was changed every 3 days. Cultures were maintained in a humidified air conditioning 5% CO_2 at 37°C. The cultured hippocampal neurons on DIV 11-12 were used for following experiments.

1.3 Whole-cell recording of NMDA-activated currents

Whole-cell voltage clamp experiments were conducted utilizing an MultiClamp 700A amplifier (Axon Instruments, Foster City, CA) at room temperature on DIV11-12 neurons. The extracellular control solution consisted of (in mmol L^{-1}): NaCl 150, KCl 3, HEPES 10, $CaCl_2 \cdot 2H_2O$ 3, Glucose 8 (pH 7.4 adjusted with 1 mol L^{-1} NaOH, and osmolarity 300–310 mOsm adjusted with sucrose). NMDA was dissolved in bath solution immediately before use. I_{NMDA} was recorded via a glass microelectrode filled with pipette solution containing (in mmol L^{-1}): CH_3O_3SCs 135, NaCl 8, HEPES 10, EGTA 0.5, Mg-ATP 4, and Na-GTP 0.3, adjusted to pH 7.2 and 305 mOsm. Being filled with pipette solution, the microelectrode had a resistance of 5–8 MΩ. The junction potential between microelectrode pipette and bath solution was −9.9 mV. This value was calculated using the junction potential calculation system of Clampex 8.0 of pCLAMP 8.0, and nulled just before forming giga-seal. In most experiments, series resistance (Rs) before compensation was 10–20 MΩ. Routinely, 70%–80% of the Rs were compensated. Only neurons with Rs less than 20 MΩ were selected for further tests. All NMDA currents were recorded while membrane potential was held at −70 mV.

To observe the influence of glycine and other agents on I_{NMDA}, NMDA, glycine and other agents were all dissolved in bath solution and applied to each neuron via a rapid drug perfusion apparatus (DAD-8VC, ALA Scientific Instrument Inc., New York, USA). This perfusion system applies pressurized injection, and allows complete exchange of solutions in the vicinity of neuron with dead volume less than 1 μL and without cross contamination and loss of mechanical stability. The tip (100 μm inner diameter) of micromanifold was usually placed 50–100 μm away from the cell recorded. The bath was continuously perfused with normal bath solution throughout all experimental procedures except application of NMDA and other agents. I_{NMDA} was recorded at an ambient temperature of 23–25°C.

1.4 Intracellular Ca^{2+} imaging

For intracellular free Ca^{2+} imaging, Fluo-3/AM was used as fluorescent Ca^{2+} indicator. Fluo-3/AM was dissolved in DMSO with final work concentration of DMSO 0.1%. On DIV 11-12, the cultured hippocampal neurons were loaded with 5 μmol L^{-1} Fluo-3/AM for 45 min at 37°C in bath solution. After being washed three times with new bath solution, the neurons were incubated at 37°C for another 30 min to complete deesterification of Fluo-3/AM. Intensity of flu-

orescence with excitation wavelength at 485 nm and emission wavelength at 525 nm was recorded every 10 s for 5 min using a laser scanning confocal microscope (TCS SP2, Leica Microsystems, Heidelberg, Germany). All image data were collected and analyzed with Leica control software of the microscope. The increase in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was determined according to the following equation: $[Ca^{2+}]_i$ increase $(\%)=(F_{525}-F_{base,525})/F_{base,525}\times100$, where F_{525} is the fluorescence intensity measured after each treatment, and $F_{base,525}$ basal fluorescence intensity.

1.5 Statistical analysis

The software Clampfit 8.0 (Axon Instruments, Forster City, CA, USA) and SigmaPlot 2001 (Jandel Scientific, Costa Madre, CA, USA) were applied for data analysis and plotting. All data are presented as mean±standard deviation. Data were analyzed with one-way ANOVA and *post hoc* Newman-Keuls test. Differences were considered statistically significant at a level of $P<0.05$.

2 Results

2.1 Dose dependent influence of glycine on high concentration NMDA-elicited currents

To explore the influence of glycine on NMDA currents, the cultured hippocampal neurons were superfused with 300 μmol L^{-1} NMDA containing different glycine concentration (0.03–10 μmol L^{-1}), current responses (I_{NMDA}) were evoked by whole cell patch clamp recordings. All neurons for recording were clamped near the resting potential in Mg^{2+}-free extracellular solution. Peak current was reduced dose-dependently by glycine when the hippocampal neurons were exposed to 300 μmol L^{-1} NMDA (Figure 1), with the mean value of peak current being 0.62%±0.05%, 0.55%±0.05%, 0.48%±0.05% of control (NMDA alone) when glycine was used at 1, 3, 10 μmol L^{-1} ($P<0.01$, Figure 1B), respectively. Examples of glycine effect on the peak current of I_{NMDA} were shown in Figure 1A with the use of 300 μmol L^{-1} NMDA. It is suggested that glycine induces an inactivation of NMDARs when NMDA is used at 300 μmol L^{-1}.

2.2 Inhibitory influence of glycine on 300 μmol L^{-1} NMDA-elicited Ca^{2+} influx

To verify the above results obtained with whole-cell recording technique, we measured calcium accumulation changes in hippocampal neurons activated by 300 μmol L^{-1} NMDA in the presence of 0.03–10 μmol L^{-1} glycine by free Ca^{2+} imaging. Identical to the results of I_{NMDA} recording shown in Figure 1, glycine decreased Ca^{2+} influx dose-dependently when 300 μmol L^{-1} NMDA was applied to the neurons (Figure 2A). With addition of NMDA at 300 μmol L^{-1}, the mean value of Ca^{2+} influx was decreased by glycine from 108.6%±7.8% of control (NMDA alone) to 55.3%±2.3% of glycine at 10 μmol L^{-1} ($P<0.01$, Figure 2B). We also found that the peak of $[Ca^{2+}]_i$ induced by NMDA decreased in a dose-dependent fashion, similar to the mean value variation. Therefore, Ca^{2+} imaging results also indicate that glycine induces an inactivation of NMDARs when NMDA is used at 300 μmol L^{-1}.

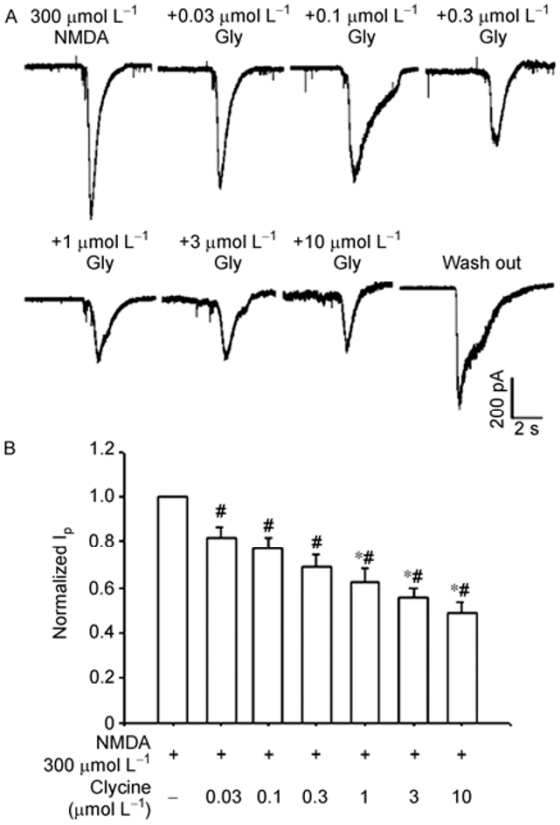

Figure 1 Dose-dependent effect of glycine on 300 μmol L^{-1} NMDA-activated peak current. A, Examples of glycine effect. B, Inhibitory effect of glycine on the peak current of I_{NMDA} elicited by 300 μmol L^{-1} NMDA (n=8). #, $P<0.01$, vs. control current elicited by 300 μmol L^{-1} NMDA without addition of glycine; *, $P<0.01$, vs. +Gly 0.03 μmol L^{-1}.

2.3 Zinc reverses the inhibitory effect of glycine on 300 μmol L^{-1} NMDA-elicited receptor activity

It is well known that the composition of the subunits of NMDARs will influence the function of the receptors. We therefore determined the expression of NR2 subunits on the cultured hippocampal neurons with immunofluorescent method. The hippocampal neurons we cultured expressed both NR2A and NR2B subunits of NMDARs on DIV 11-12 (data not shown). To verify the involvement of NMDAR subunits, we used two inhibitors of NR2A and NR2B subunits, Zn^{2+} and ifenprodil respectively [2]. As a result, in the presence of $ZnCl_2$ (30 nmol L^{-1}), an inhibitor of NR2A subunit, 300 μmol L^{-1} NMDA-elicited Ca^{2+} influx was ele-

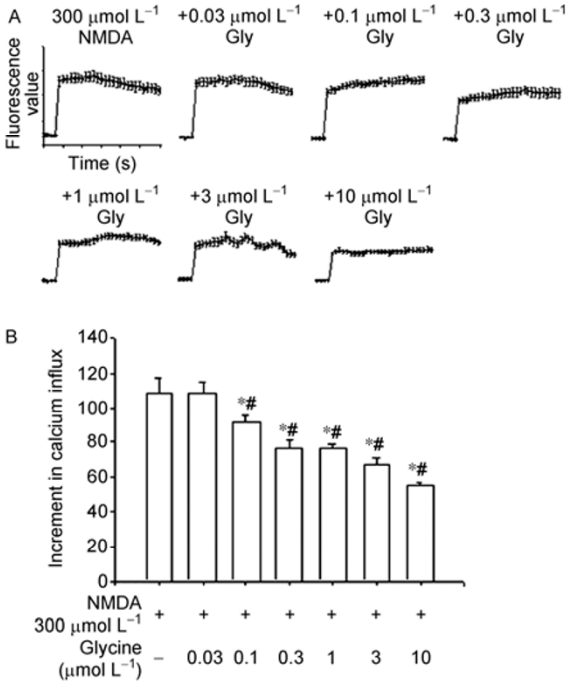

Figure 2 Dose-dependent effect of glycine on 300 μmol L^{-1} NMDA-elicited Ca^{2+} influx (n=20). A, Examples of glycine effect. B, Inhibitory effect of glycine on 300 μmol L^{-1} NMDA-elicited Ca^{2+} influx. #, P<0.01, vs. NMDA without addition of glycine; *, P<0.01, vs. +Gly 0.03 μmol L^{-1}.

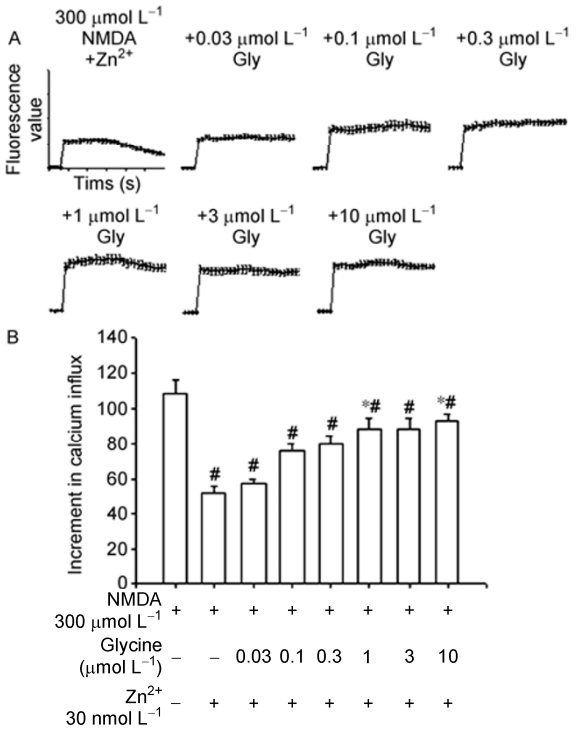

Figure 3 Effect of glycine on 300 μmol L^{-1} NMDA-elicited Ca^{2+} influx in the presence of 30 nmol L^{-1} $ZnCl_2$ (n=20). A, Examples of glycine effect. B, Mean values of Ca^{2+} influx. #, P<0.01, vs. 300 μmol L^{-1} NMDA alone; *, P<0.01, vs. NMDA+Zn^{2+}(30 nmol L^{-1}) without addition of glycine.

vated dose-dependently by glycine from 52.1%±4.1% at control (NMDA alone) to 92.9%±3.6% of glycine at 10 μmol L^{-1} (P<0.01, Figure 3). However, in the presence of ifenprodil (10 μmol L^{-1}), an inhibitor of NR2B subunit, NMDA-elicited Ca^{2+} influx were reduced, but glycine still dose-dependently reduced NMDAR responses induced by 300 μmol L^{-1} NMDA, the mean value of Ca^{2+} influx was decreased by glycine from 66.4%±4.0% (NMDA with ifenprodil alone) to 47.1%±3.3% of glycine at 10 μmol L^{-1} (P<0.01, Figure 4). Comparing the influence of glycine on NMDA-elicited Ca^{2+} influx with or without ifenprodil, there was only some difference in declining degree, but the decrease tendency was not reversed. Quantify the calcium influx changes of glycine inhibitory effects on NMDAR activity after use of $ZnCl_2$ or ifenprodil, it was found that addition of $ZnCl_2$ reversed the dose-dependent inhibitory effect of glycine on 300 μmol L^{-1} NMDA-induced Ca^{2+} influx, but ifenprodil did not change the tendency of inhibitory influence of glycine on Ca^{2+} influx (Figure 5).

Furthermore, we confirmed the reversal efficiency mediated by zinc on the inhibitory effect of glycine at 300 μmol L^{-1} NMDA using whole cell recordings. As shown in Figure 6, glycine dose-dependently still reduced whole-cell currents elicited by 300 μmol L^{-1} NMDA with pretreatment of NR2B inhibitor, 10 μmol L^{-1} ifenprodil (Figure 6B), while 50 nmol L^{-1} Zn^{2+} exerted notable different tendency on the glycine-dependent inhibition of NMDA currents, it

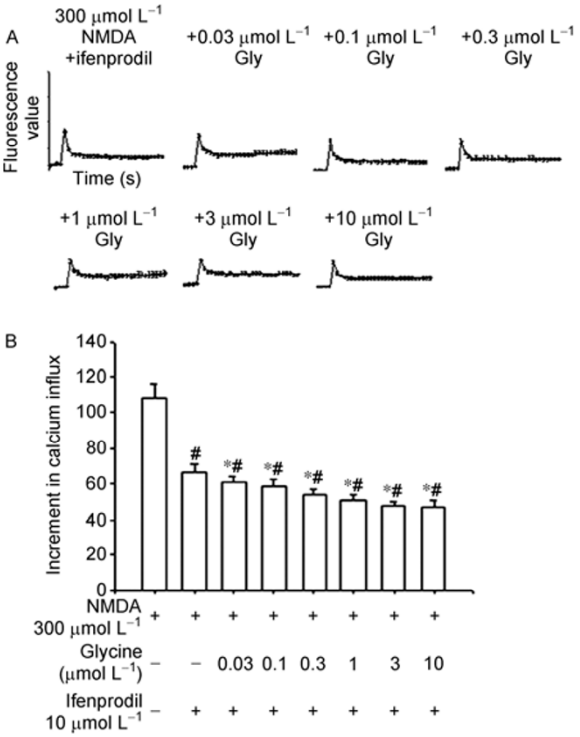

Figure 4 Effect of glycine on 300 μmol L^{-1} NMDA-elicited Ca^{2+} influx in the presence of 10 μmol L^{-1} ifenprodil (n=20). A, Examples of glycine effect. B, Mean values of Ca^{2+} influx. #, P<0.01, vs. 300 μmol L^{-1} NMDA alone; *, P<0.01, vs. NMDA+ifenprodil without addition of glycine.

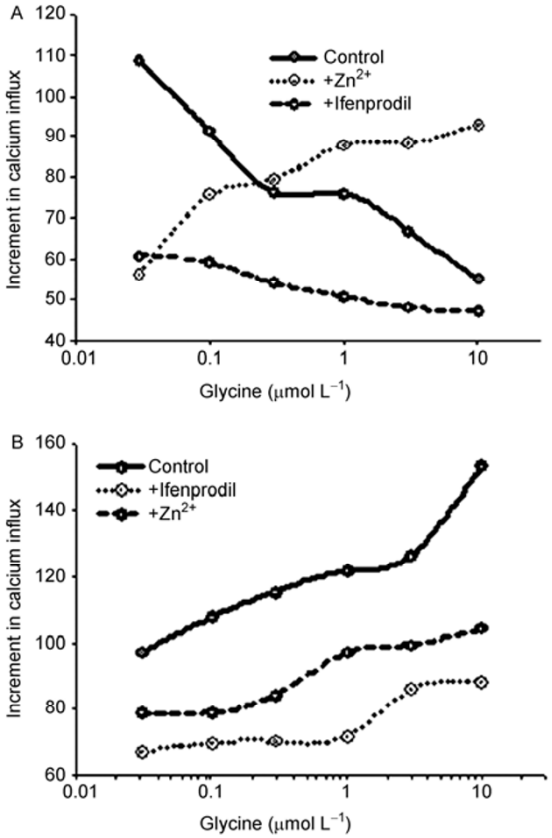

Figure 5 Dose-response curve of glycine on 300 μmol L^{-1} NMDA-elicited Ca^{2+} influx in the presence or absence of NMDAR inhibitor (n=20). A, $ZnCl_2$, a NR2A blockor, was used at 30 nmol L^{-1}. B, Ifenprodil, a NR2B inhibitor, was used at 10 μmol L^{-1}.

reversed the glycine dose dependent inhibition of I_{NMDA} (Figure 6A). Hence, NR2A subunit is potentially responsible for the glycine-dependent inactivation of NMDAR or is capable of modulating the glycine-dependent NMDAR inactivation.

3 Discussion

NMDARs are a principal subtype of excitatory ligand-gated ion channel with prominent roles in physiological and disease processes in the central nervous system. Recognition that modulating mechanisms of NMDAR activity was profoundly changed our understanding of chemical synaptic communication in the central nervous system. Ca^{2+}-dependent inactivation is a reversible decrease in peak current which can be induced with a rise in extracellular Ca^{2+} concentration without agonist activation of the receptor itself. Desensitization is a decrease in the current response induced in the persistent presence of a glutamate site agonist [17–19]. Glycine-dependent inactivation, a new mechanism raised in our previous study, implies dual effects of glycine on NMDAR activation in hippocampal neurons via impacting glycine binding site affinity of NMDARs [13].

Up to now, molecular cloning of the cDNAs of individual NMDAR subunits has distinctly indicated that functionally and pharmacologically distinct receptor subtypes could be generated from the different combinations of the NR1 and NR2 subunit family [20]. Desensitization characteristics of NMDARs, which depends on the subunits expressed by the neurons, may be remarkably changed from one neuron to another. Accumulating evidence shows that there are subunit-specific differences in the desensitization properties of NMDARs [7,21]. The issue whether this glycine-dependent inactivation also appears in NMDAR complex composed of NR1 with other NR2 subunits remains to be elucidated. The study by Kendrick *et al*. has suggested that NR2A-like and NR2B-like NMDAR subtypes can exist within a single hippocampal neuron and can be selectively activated by altering the concentration of glycine [22]. In order to clarify these issues, we tested the glycine-dependent inactivation in the presence of NMDAR inhibitors, Zn^{2+} and ifenprodil respectively for NR2A and NR2B subunits [2,23]. The present study documented that blocking of NR2A subunit by Zn^{2+} reversed the inhibitory effect of glycine on 300 μmol L^{-1} NMDA-induced NMDAR responses, but ifenprodil, a NR2B subunit inhibitor, failed to change this type of inhibitory glycine effect. Our results suggested that NR2A subunit of NMDARs in hippocampal neurons was potentially responsible for this glycine-dependent inactivation, which was a novel inhibitory modulation of glycine on NMDAR activity.

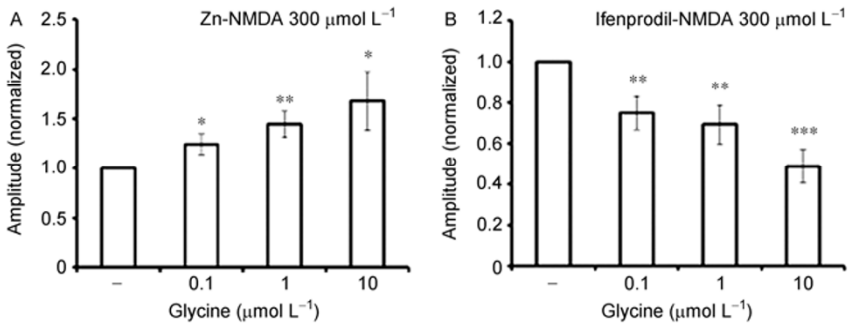

Figure 6 Dose-dependent effect of glycine on 300 μmol L^{-1} NMDA-elicited peak current (n=10). A, In the presence of NR2A inhibitor Zn^{2+} ($ZnCl_2$, 50 nmol L^{-1}). B, In the presence of NR2B inhibitor ifenprodil (10 μmol L^{-1}). *, P<0.05; **, P<0.01; ***, P<0.001, vs. Gly (glycine) 0 μmol L^{-1}.

NMDARs containing the NR2A subunit exhibit high affinity voltage-independent inhibition by Zn^{2+} [23]. The molecular determinants of high affinity zinc binding have been demonstrated to lie in the amino terminal domain of the NR2A subunit [24]. Studies of Gielen *et al*. [25] and Erreger *et al*. [26] suggest an allosteric interaction between Zn^{2+} and glutamate binding domains on NR2A. In other words, the affinity of zinc for its binding site is regulated by glutamate binding, thus causing desensitization or allosteric inhibition of NMDARs. Nahum-Levy *et al.* indicate an influence of small cations (not including Zn^{2+}) on glutamate affinity but not glycine agonist affinity for NMDAR. Why could Zn^{2+} reverse the glycine-dependent inactivation of NMDAR activity, as shown in the present study? We assumed that Zn^{2+} binding to NR2A subunit might cause an allosteric modulation of glycine site on NMDARs in addition to an influence on the glutamate binding site. Nong *et al.* show that glycine binding to its site on NMDARs prime but does not directly induce NMDAR internalization, and that subsequent application of NMDA or glutamate elicits NMDAR internalization and resultant reduction of NMDA currents [10]. Clathrin-dependent endocytosis of the NMDAR complex that was initiated by synaptic released glutamate in the presence of 100 μmol L^{-1} glycine has also been proposed [27]. These reports inspire us to investigate the mechanism underlying the glycine-dependent inhibition of NMDAR activity.

Modulation of the NMDAR response is an important mechanism for the physiological or pathophysiological consequences of NMDAR activation [28–30]. Concentrations of glutamate, glycine or D-serine and many other factors (including Zn^{2+}, Mg^{2+}, H^{+}, etc.) at the synaptic level of the neurons are important determinants of the ion current flowing through the NMDAR-gated ion channel. Recently, Takeda *et al.* report that Zn^{2+} promote NMDAR-dependent long-term potentiation (LTP) at hippocampal CA1 synapses [31,32]. Our present results may at least in part explain the underlying mechanism. Tetanic stimulation will greatly elevate the concentration of glutamate in the synaptic cleft, if glycine-dependent inactivation of NMDARs is accomplished through clathrin-dependent receptor internalization, NMDA currents will not be potentiated, and LTP will be inhibited. The presence of Zn^{2+}, which is co-released with glutamate, will reverse this effect of glycine on NMDARs through inhibiting receptor internalization initiated by NMDAR activation which is induced by high level of glutamate.

In conclusion, the present study suggested that zinc could reverse glycine-dependent inactivation which occurred when NMDARs are activated by high concentrations of NMDA. This modulation of glycine inhibitory effect on NMDARs implies a novel approach to the regulation of synaptic plasticity in long-term potentiation, learning, and memory.

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